LOW–TEMPERATURE, LONG–TIME HEATING OF BOVINE MUSCLE
3. Collagenolytic Activity

SUMMARY—Naturally occurring collagenolytic activity was found in the water-soluble fraction of bovine muscle. General proteolytic activity determined with Azocoll indicated that this total activity was much greater than the collagenase activity specifically determined according to the method of Wünsch and Heidrich. The collagenase fraction was concentrated by polyacrylamide gel electrophoresis and the activity of the enzyme was studied under various pH and temperature conditions. This collagenase could remain active in the meat at cooking temperatures experienced in long-time, low-temperature cooking, $<60°C$. With faster heating and higher internal temperatures, $>70-80°C$, the collagenase observed in this study is inactivated.

INTRODUCTION

BY DEFINITION collagenase is an enzyme attacking native collagen at or near physiological pH (Mandl, 1961). However, collagen is easily denatured and then is susceptible to almost any unspecific proteolytic enzyme.

Proteolytic enzymes are present in muscle (Whitaker, 1964) and the pH optima of some of them are within the range of meat. Koszalka et al. (1960) reported a proteolytic enzyme in rat skeletal muscle having optimal activity at pH 8.5–9.0. Nogochi et al. (1966) found that this enzyme is located mainly in the myofibrils.

Davey et al. (1968) describe the aging of meat as a loss of the tensile strength of the myofibrillar components of the muscle cell brought about by disintegration of the Z bands. This dissolution could lead to a change in the extractability of proteins.

Solovyov et al. (1967) isolated muscular cathepsins at pH 5.6. This complex of enzymes possessed a relatively low but clearly pronounced elastase activity. When studying catheptic action on the pure elastin obtained from fresh warm meat, they found more than a threefold increase in the N-terminal residues of the 6 amino acids investigated. Simultaneously, soluble products of elastin breakage appeared. There was a 68% increase of N-terminal amino acids. The amount of elastolysis-soluble products had a nearly twofold increase during meat aging.

Gross et al. (1965) reported a collagenolytic enzyme in anuran tadpole tissue cultures. This enzyme has a pH optimum of 6–8, loses activity upon heating for 10 min at 50–60°C and is inhibited by EDTA and cysteine. Lazarus et al. (1968) extracted a collagenase operative at neutral and alkaline pH from the granule fraction of human granulocytic leukocytes. Woods et al. (1965) demonstrated far larger collagenolytic activity in the bone of rat than in any other of the tissues studied; namely, kidney, leukocytes, brain and liver.

The objective of this study is to determine whether collagenolytic activity could be measured in meat and to relate any such activity to changes occurring in bovine muscle during low-temperature cooking.

EXPERIMENTAL

LONGISSIMUS, rectus femoris and semitendinosus muscles from Hereford steers were heated at 0.1°C/min to holding temperatures of 37, 45 and 60°C. Samples were withdrawn periodically for analysis. Both the exuded meat juice and the water-soluble fractions were freeze dried and held frozen until used in the present study. Details on muscle preparation, heating program and extraction of the water-soluble material were presented in a preceding paper (Laakkonen et al., 1969).

Collagenolytic activity

Collagenolytic activity of both the water-soluble fraction and the drip from muscles given various heat treatments was determined quantitatively according to a modification of the method by Wünsch et al. (1963), using 4-phenyl-azo-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (Mann Research Laboratories, Inc., 136 Liberty St., N.Y. 10006) as substrate.

40 mg of the freeze-dried sample were dissolved in 0.5 ml of 0.01 M calcium acetate. 10 mg substrate was dissolved in 0.1 ml methanol and diluted to 10 ml with acetate-veronal buffer, pH 7.7. 3 identical tubes were preincubated at 37°C for 15 min, and 3 additional tubes kept at 0°C. The substrate solution was similarly preincubated, then 2 ml was mixed with each of the protein solutions. After holding for 15 min, 0.5 ml of each reaction mixture was pipetted into 1 ml of 0.5% citric acid then 5 ml ethylacetate added. The mixture was shaken for 15 sec. The phases were allowed to separate and 4 ml of the ethylacetate phase were removed and dried over 0.3 g of anhydrous sodium sulfate. The difference in absorbance at 320 mμ between each sample incubated at 37° C and an average sample incubated at 0°C was used to calculate collagenase activity in γ collagenase/mg dried sample from a standard curve. This standard curve was established using 4-phenyl-azo-benzyloxycarbonyl-prolyl-leucine in ethylacetate. The samples incubated at 0°C were used as the reference because preliminary experiments showed that the ethylacetate also extracted various amounts and types of meat colors, depending upon the sample and heat treatment.

Proteolytic activity

Azocoll (Calbiochem, Box 54282, Terminal Annex, Los Angeles, California) is an insoluble but hydrophilic complex of collagen and an azo dye (Schubert et al., 1968). Proteolytic enzymes release the dye from the complex, the rate of release reflecting the proteolytic activity of the sample.

40 mg of the freeze-dried sample were dissolved in 5 ml of 0.1 M sodium phosphate buffer, pH 7.5, at 0°C. Then 25 mg Azocoll were added and the mixture incubated for 15 min. Triplicate preparations were incubated at both 37 and 0°C. After incubation, each sample was filtered and the absorbance of the filtrate at 580 mμ was determined. The difference between the absorbance of each solution incubated at 37°C and the absorbance of an average solution incubated at 0°C, indicative of the dye released by the enzymic activity of the sample, was converted to collagenase activity using a standard curve supplied by the substrate manufacturer. The samples incubated at 0°C were used as references since myoglobin, λ max = 555 mμ, and oxymyoglobin, λ max = 575–585 mμ, are both soluble under these conditions.

Isolation and characterization of collagenolytic activity

The freeze-dried water-soluble fraction obtained from rectus femoris heated at 0.1°C/min for 4 hr to 52°C was chosen for the attempts to characterize the collagenolytic activity. 40 mg of the dried material were dissolved in 250 μl of 0.1 M calcium acetate and stored at 0°C for 2 hr. The solution was then evenly distributed among 20 slots across the width of a polyacrylamide gel and subjected to electrophoresis. Details of the electrophoresis have already been described. The unstained gel was cut transversely into 10-mm-wide segments, 11 altogether. The strong myoglobin band reported previously (Laakkonen et al., 1969) appeared in the 6th segment from the origin. Each segment was soaked in 10 ml of 0.1 M calcium acetate at 0°C. After a 5-day soaking period, 0.5-ml portions of the solution were subjected to the Wünsch et al. (1963) collagenase assay.

Triplicate determinations of the collagenolytic activity of material dissolved from the 7th and 8th strips were made at pH 4, 6, 7, 8, 9 and 10. The acetate-veronal buffer used was

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adjusted to the desired pH values with 0.1 N HCl or NaOH.

3 to 5 determinations at pH 7.7 of the collagenolytic activity dissolved from the same gel strips were made at each of 3 incubation temperatures: 37, 45 and 60°C.

RESULTS & DISCUSSION

COLLAGENOLYTIC activity, as shown in Figure 1, was detected in all 3 muscles examined and was not completely eliminated by heating the meat to 58–60 °C. The activity detected in the water-soluble fraction decreased as heating progressed. The longissimus lost collagenolytic activity more rapidly during the early stages of heating, whereas the semitendinosus lost activity in the later stages of heating. The exuded meat juices (drip) had higher collagenolytic activities than the corresponding water-soluble fraction except for the longissimus heated to 58–60 °C.

One reason for the higher activities in the drip may be that in the cut surface of the muscle there may be a mechanism similar to that which releases collagenase activity in healing wounds (Ross, 1968, and Grillo, 1967). Another reason may be a greater increase in calcium ion in the drip than in the meat, as calcium ion is an activator of collagenase. Van den Berg et al. (1964) found increasing amounts of calcium in meat juices exuded during cooking of poultry meat.

Houck et al. (1968) reported that when whole normal skin excised from rats had been incubated in sterile-organ culture at 56°C for 16 hr, this in vitro burned tissue demonstrated a translation of 30% of the insoluble collagen to a form extractable in 0.15 M NaCl. The normally bound, inactive collagenase is converted to a free, active form. This conversion is associated with the release of proteolytic activities. A similar release of collagenase may occur in muscle tissue during aging and during low-temperature heating. The calcium ion may play a role, since Webb et al. (1967) reported a 3X increase in extractable calcium after 9 days of aging at 3.3°C.

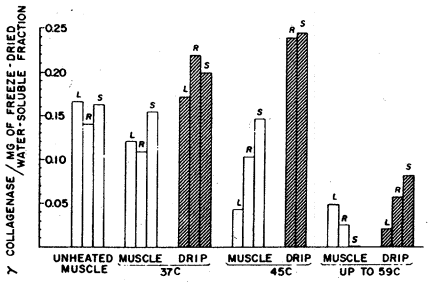

Fig. 1—Amounts of collagenase in the freeze-dried water-soluble fraction and in the freeze-dried exuded meat juice (drip) from unheated muscles, and after 6 hr of heating at a rate of 0.1°C/min to 37, 45 and 59°C. L= longissimus; R = rectus femoris; S = semitendinosus.

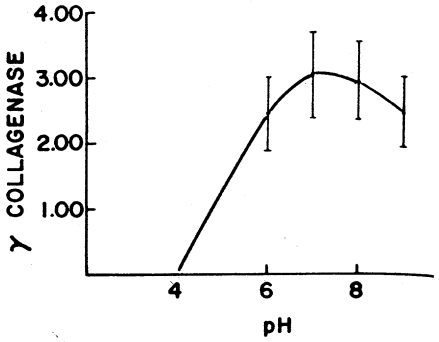

Fig. 2—Collagenase activity at 37°C at differen pH values of the 0.1 M calcium acetate solutio obtained by soaking collagenase-active poly acrylamide gel strips. Activity is expressed as γ collagenase in 0.5 ml of 0.1 M calcium acetat solution. Small vertical lines indicate the stand ard deviations of 3 to 5 determinations.

The relatively high amount of collagenolytic activity after heating for 6 hr at 37 and 45°C, and the rapid decrease upon heating to 60°C may explain Cover's (1941) early finding that heating for 23 hr in a 90° C oven gave shear values of 1/2 to 1/3 those obtained by heating for 3 hr in 90°C water. The slower penetration of heat causes less loss of meat juice; therefore, more collagenolytic activity will be retained in the meat.

Table 1 shows the results of the determination of proteolytic activities, reported as γ collagenase/mg dried sample. These values are about 10 times those obtained using the specific collagenase substrate of Wünsch et al. (1963). This wide difference confirms the expectation that proteolytic enzymes other than collagenase also are present. The total proteolytic activity did not change greatly upon heating to 37°C for 6 or 10 hr. Heating for 4 hr to 50.5°C internal temperature seemed to increase the proteolytic activity, although not in all samples. Heatin for 6 hr to a 58.5°C internal temperatur caused a clear decrease in activity.

The pH activity curve obtained for th collagenolytic material concentrated b electrophoresis is shown in Figure 2. Th pH optimum seemed to be about pH 7– and there was no activity at pH 4. Thes results are consistent with those reporte by Lazarus et al. (1968). The temperatu activity curve is shown in Figure 3. Of th 3 temperatures actually studied, the en zyme was most active at 37°C, but wa significantly active at 45°.

There is probably little collagenolyti activity in meat during aging when the pH is around 5.5 and the temperature is clos to 0°C. This may be the reason collage nase has not been reported in meat.

Results of the present study seem t show that in aged meat there is a collage

Table 1—Proteolytic activity in freeze-dried water-soluble extracts of heated muscle samples as measured with Azocoll and expressed as gamma quantities of collagenase.

Muscle sample	Heating		Collagenase activity present per mg of freeze-dried water-soluble fraction (gamma)
	Temperature (C)	Time (hr)	
Rectus femoris, right[1]	—	0	1.36
Rectus femoris, left[1]	—	0	1.38
Rectus femoris[2]	37	6	1.24
Rectus femoris[1]	37	10	1.54
Rectus femoris[1]	52	4	2.46
Longissimus dorsi[1]	52	4	1.50
Semitendinosus[1]	52	4	1.60
Rectus femoris, drip[1]	60	6	.75

[1] Steer A.
[2] Steer B.

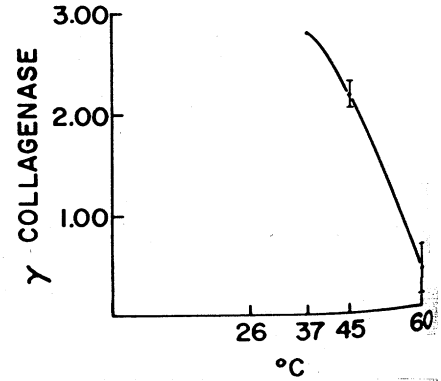

Fig. 3—Collagenase activity at pH 7.7 at diffe ent temperatures of the 0.1 M calcium aceta solution obtained by soaking collagenase-acti polyacrylamide gel strips. Activity is expres as γ collagenase in 0.5 ml of 0.1 M calciu acetate solution. Small vertical lines indica the standard deviations of 3 to 5 determin tions.

nase-like enzyme capable of attacking the peptide chain L-prolyl-L-leucyl-glycyl-L-prolyl between leucine and glycine. Neither trypsin, chymotrypsin, carboxypeptidase A & B nor amidase has this potential (Wünsch et al., 1963). In most meat-cooking methods, however, the rate of rise in temperature is fast and when the final internal temperature of 70–80 °C is reached this enzyme is obviously inactivated. If meat is heated slowly to an internal temperature of 60°C, this collagenase-like enzyme is probably capable of producing tender meat.

Once the triple helix of collagen is unfolded, several other proteolytic enzymes besides collagenase may attack it. Results appearing in Table 1 indicate that proteolytic activity in addition to collagenase was much greater than the collagenase activity specifically determined according to the method of Wünsch et al.

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